REMARKS

Claims 9 and 11-14 are pending. Favorable reconsideration is respectfully requested.

Applicants would like to thank Examiner Slobodyansky for the helpful and courteous discussion held with their representative on November 16, 2006. During the discussion, amending the claims to overcoming the outstanding rejections was discussed. The following remarks expand on the discussion with the Examiner.

The rejection of the claims under 35 U.S.C. §103(a) over Laird et al. or Thaller et al. alone or in view of Cowman et al. is respectfully traversed.

In the *E. coli* mutant strain (54G2) disclosed by Laird et al., the purEK gene is disrupted in addition to the ushA and aphA genes. However, Laird et al. do not disclose accumulation of nucleoside 5′-phosphate esters in the medium by culturing the 54G2 strain. Further, the disruption of the purEK gene imparts a purine auxotrophy to the strain. Since the purEK gene codes for 5-phosphoribosyl-5-aminoimidazole carboxylase involved in purine biosynthesis, the 54G2 strain cannot produce nucleoside 5′ -phosphate esters such as inosine 5′-phosphate ester (IMP). Thus, the claimed method for producing nucleoside 5′-phosphate ester is not obvious over Laird et al.

Generally, bacteria have around 5 different types of 5′-nucleotidases. For example, Shiio and Ozaki (J. Biochem., 83, 409-421 (1978); a copy is submitted herewith) revealed that *Bacillus subtilis* has 7 types of 5′-nucleotidases. See Table V of the reference. It had been expected that *E. coli* also has around 5 types of 5′-nucleotidases, and it had been unknown which one of such 5′-nucleotidases should be disrupted so that accumulation of nucleosides 5′-phosphate esters increases. However, the Inventor of the present invention revealed that *E. coli* has only two types of 5′-nucleotidases (AphA and UshA), encoded by aphA and ushA genes (Table 5 and page 32, lines 4-6 of the present specification). In

addition, when either the aphA gene or ushA gene was disrupted, i.e., only one of the two genes was disrupted, IMP was not accumulated in the medium (Table 6 of the present specification). On the other hand, when both the aphA gene and the ushA genes were disrupted, and average of 0.8g/L of IMP was accumulated in the medium (Table 6). As described above, it was revealed through the experiment that disruption of either the aphA gene or the ushA gene was not effective in accumulation of IMP, whereas disruption of both the aphA gene and the ushA gene was effective in accumulation of IMP. These results would never have been obvious from Thaller et al. alone or in view of Cowman et al. Therefore, the method for producing nucleoside 5′-phosphate ester of the present application is not obvious from Thaller et al. alone or in view of Cowman et al.

In view of the foregoing, withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, first paragraph, is believed to be obviated by the amendment submitted above. Accordingly, withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, second paragraph, is believed to be obviated by the amendment submitted above. Therefore, withdrawal of this ground of rejection is respectfully requested.

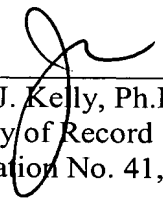
Applicants submit that the present application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Customer Number
22850

Tel: (703) 413-3000
Fax: (703) 413 -2220
(OSMMN 06/04)



James J. Kelly, Ph.D.
Attorney of Record
Registration No. 41,504

J. Biochem. 83, 409–421 (1978)

Cellular Distribution and Some Properties of 5′-Nucleotidases in *Bacillus subtilis* K

Isamu SHIIO and Hachiro OZAKI
Central Research Laboratories of Ajinomoto Co.,
Kawasaki-ku, Kawasaki, Kanagawa 210

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The distribution of 5′-nucleotidases of *Bacillus subtilis* K No. 231, an inosine-producing bacterium, was investigated. It was found that this organism has an extracellular 5′-nucleotidase as well as various cellular 5′-nucleotidases, which include a major one bound to the cell wall and minor ones in the cytoplasm and on the cytoplasmic membrane. The cell wall-bound 5′-nucleotidase was specifically solubilized by treatment of the sonic cell debris with lysozyme, but not by treatment with various other hydrolyzing enzymes, detergents or high concentrations of inorganic salts. The extracellular enzyme was more repressible by inorganic phosphate than the cellular ones. Among the three cellular 5′-nucleotidases the cytoplasmic enzyme was the most repressible by inorganic phosphate. The crude preparations of the extracellular, cell wall-bound and cytoplasmic 5′-nucleotidase showed single peaks of activity on gel filtration, at the elution volumes corresponding to molecular weights of 67,000, 900,000 and 600,000, respectively. Upon DEAE-cellulose column chromatography of the peak fractions of the three preparations, two peaks (E_1 and E_2), three peaks (W_1, W_2, and W_3) and two peaks (C_1 and C_2) were obtained, respectively. The molecular weights of E_1, E_2, W_1, W_2, W_3, C_1, and C_2 were determined to be 27,000, 46,000, 37,000, 21,000, 640,000, 38,000, and 260,000, respectively. All seven enzymes showed optimum pH between 7.0 and 8.0, and specifically hydrolyzed the phosphomonoester bonds of purine nucleoside-5′-monophosphates but not those of 5′-pyrimidine nucleotides. The cellular enzymes showed slight activity toward UDP-glucose whereas the extracellular enzymes did not. None of the enzymes hydrolyzed 4-nitrophenylphosphate or bis(4-nitrophenyl)phosphate. All the enzymes showed the same K_m values, 1.5–1.9 μM for AMP. High concentrations (0.1 M and 1 M) of NaCl, KCl, and $(NH_4)_2SO_4$ activated the cellular 5′-nucleotidases more strongly than the extracellular enzymes. Mg^{2+} and Mn^{2+} stimulated the activities of the cytoplasmic enzymes, C_1 and C_2, specifically. All the enzymes were inhibited by Zn^{2+}, Cu^{2+}, ATP, and PO_4^{2-}. The PO_4^{3-} inhibition was of mixed type with respect to AMP, and the inhibitor constant for PO_4^{3-} was almost the same, 0.4–1.1 mM, for all the enzymes.

Previous studies on the regulatory mechanism for purine biosynthesis in *Bacillus subtilis* have shown that genetic or physiological release from regulation causes the excretion of various purine nucleosides, but not of the nucleotides (*1*, *2*). Since the purine derivatives synthesized *de novo* are 5′-nucleotides

in this organism (*3*), as in pigeon liver, the excreted nucleosides are presumably formed by hydrolysis of the corresponding nucleotides by 5′-nucleotidases [EC 3. 1. 3. 5]. Moreover, preliminary experiments also showed that adenine auxotrophs of this organism grew on either AMP or adenine and that their mutants, which did not grow on AMP but did on adenine, had much lower 5′-nucleotidase activities than the parents, suggesting that the cell membrane of this organism may not be permeable to 5′-nucleotides but may be to the nucleosides. Since these observations suggest an important role of 5′-nucleotidase in the extracellular accumulation of the nucleosides, it was of interest to study this enzyme of *B. subtilis* K.

Although 5′-nucleotidase activity has been found in the culture fluid as well as in washed or sonically disrupted cells of *B. subtilis* (*4–6*), no attempt has been made to purify these enzymes, or to compare their properties. On the other hand, a purified nucleoside diphosphate sugar hydrolase of *B. subtilis* W-23 has been reported to have 5′-nucleotidase activity (*7*). However, no evidence is available as to whether this enzyme is the only, or even the major, 5′-nucleotidase activity in this organism.

The present paper deals with the distribution in the culture broth, partial purification and comparison of some general properties of 5′-nucleotidases of *B. subtilis* K No. 231, a bacterium which accumulates a large amount of inosine under conditions of adenine limitation.

MATERIALS AND METHODS

Bacterium—*Bacillus subtilis* K No. 231, an inosine-producing adenine auxotroph (*1*), was used.

Culture Media—Medium-1 was a nutrient broth, Medium-1 in the previous paper (*1*), while Medium-2 was a synthetic glucose medium, Medium-3 in the previous paper (*2*), containing 100 mg of adenine. Medium-3 had the same composition as Medium-2, except that 0.2 g of KH_2PO_4 was added per liter of the medium instead of 1 g of KH_2PO_4.

Cultivation—A loopful of cells grown overnight on Medium-1 agar plates was inoculated into 20 ml of Medium-2 or Medium-3 in a 500 ml flask and aerobically cultured with shaking at 30°C for 48 h.

Chemicals—IMP was a product of Ajinomoto Co. Other 5′-nucleotides, trypsin inhibitor (soybean), lysozyme (egg white) [EC 3.4.421.], catalase (bovine liver) [EC 1. 11. 1. 6], phospholipase C [EC 3.1.4.3], and subtilisin BPN′ [EC 3.4.4.16] were purchased from Sigma. ADP-D-glucose, glucose-1-phosphate, adenosine deaminase [EC 3.5.4.4], hexokinase (yeast) [EC 2.7.1.1], and peroxidase (horse radish) [EC 1.11.1.7] were purchased from Bochringer. TES (N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid), bis(4-nitrophenyl)-phosphate, trypsin [EC 3.4.4.4], and ribonuclease [EC 2.7.7.16] were from Calbiochem. Deoxyribonuclease [EC 3.1.4.5] was from Nutritional Biochemicals Corporation. 4-Nitrophenylphosphate and chymotrypsin[EC 3.4.4.5] were from Tokyo Kasei Kogyo Co. DEAE-cellulose (0.97 meq/mg) was a product of Midorijuji Co.

Assay of 5′-Nucleotidase—Method A: The reaction mixture contained 0.1 M Tris-HCl, pH 7.2, 8 mM IMP and 5′-nucleotidase in a total volume of 1.0 ml. After incubation for 30 min at 37°C, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid. After centrifugation of the reaction mixture, inorganic phosphate in the supernatant was assayed by the method of Lowry and López (*8*). One unit of the enzyme is defined as the amount of enzyme which produces 1 μmol of phosphate per min under the assay conditions.

Method B: For studies on the kinetics or the effect of phosphate, formation of adenosine from AMP was followed by the method of Burger *et al.* (*9*); the reaction mixture contained 0.067 M Tris-HCl, pH 7.2, 0.01 mM AMP, 0.3 unit of adenosine deaminase and 5′-nucleotidase in a total volume of 1.5 ml. The reaction was started by adding 5′-nucleotidase in a cuvette with 1.0 cm light path at room temperature (21–25°C). The initial rate of increase of the absorbance at 262.5 nm was recorded on an expanded scale (0.1 absorbance, full scale) with a Gilford 240 recording spectrophotometer.

Assay of Alkaline Phosphatase—The reaction mixture contained 0.1 M glycine-NaOH buffer, pH 10.0, 8 mM 4-nitrophenylphosphate and alkaline phosphatase in a total volume of 1.0 ml. After 10 min at 37°C, the reaction was stopped by adding 1 ml of 0.5 M Na_2HPO_4. Insoluble materials, if present, were removed by centrifugation and the absorbancy at 420 nm was read. One unit of the

enzyme is defined as the amount of enzyme which produces 1 μmol of phosphate per min under the assay conditions.

Purification of 5′-Nucleotidase—From the culture supernatant: *B. subtilis* K No. 231 was grown in Medium-2 for 48 h and centrifuged off, then the culture supernatant (4 liters) was dialyzed against 40 liters of 0.05 M Tris-HCl, pH 7.5, (buffer A) for 24 h and then overnight against 12 liters of the buffer. The dialyzed solution was placed on a DEAE-cellulose column (3.5×33 cm) previously equilibrated with buffer A. After the sample had entered the column, the column was washed with 2 liters of the buffer, and eluted with a concentration gradient of NaCl formed from 1.5 liters of buffer A and 1.5 liters of buffer A containing 0.4 M NaCl. The fractions of the two 5′-nucleotidase peaks (E_1, E_2), shown in Fig. 3A, were separately pooled and concentrated by adding solid ammonium sulfate to 80% saturation, and the precipitates were dissolved in a small volume of buffer A. The fractions were separately gel-filtered through a Sephadex G-100 column (3.3×42 cm) using buffer A. The enzyme fractions were pooled and used for all experiments in this paper.

From the cells: The bacterium was grown in Medium-3 for 48 h, then the cells (19.2 g dry weight) harvested from 3,840 ml of the culture broth were washed twice with buffer A and suspended in the same buffer to give a final volume of 291 ml. The cell suspension was subjected to sonic treatment until complete disruption of the cells was confirmed by microscopic observation. The sonicate was centrifuged at 30,000×*g* for 30 min, and then at 100,000×*g* for 1 h to remove small particles. Then, solid ammonium sulfate was added to the supernatant, and the precipitate between 50% and 80% saturation of ammonium sulfate was dissolved in a small volume of buffer A and fractionated again using saturated ammonium sulfate solution. The material precipitated between 30% and 45% saturation was dissolved in a small volume of the buffer, and gel-filtered through a Sephadex G-200 column (2.64×45 cm) using buffer A. The enzyme fractions were pooled (35 ml) and chromatographed on a DEAE-cellulose column (1.5×15 cm) under the conditions described in the legend to Fig. 3B. The active fractions of the two peaks (C_1 and C_2) were pooled separately and used for experiments.

The cell debris precipitated by centrifugation of the sonicate was washed twice with buffer A, suspended in the same buffer to give a final volume of 200 ml, and then treated with lysozyme (200 mg) at 30°C for 40 min. After centrifugation at 100,000×*g* for 1 h, the supernatant was fractionated with solid ammonium sulfate. Precipitates between 60% and 80% saturation of ammonium sulfate were dissolved in a small volume of buffer A and gel-filtered through a Sephadex G-200 column (2.64×45 cm) using the same buffer. The enzyme fractions were pooled (35 ml) and chromatographed on a DEAE-cellulose column (1.5×15 cm) under the conditions described in the legend to Fig. 3C. Fractions of the three peaks (W_1, W_2, and W_3) were pooled separately and used for experiments.

Distribution of 5′-Nucleotidase and Alkaline Phosphatase in the Spheroplasts—The washed cells (260 mg dry weight) grown in Medium-3 for 48 h were incubated with 40 mg of lysozyme in 20 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 20% sucrose, 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (buffer B) at 30°C for 40 min. Then the mixture was chilled in ice, diluted with 20 ml of cold buffer B and centrifuged at 15,000×*g* for 10 min. The precipitates were washed twice with buffer B, then the spheroplasts in the precipitates were burst by incubation with 10 ml of 0.1 M Tris-HCl buffer, pH 7.5, at 0°C for 30 min and the mixture was centrifuged at 1,700×*g* to remove contaminating intact cells. The supernatant was then centrifuged at 30,000×*g* for 30 min. The precipitates obtained were washed twice with buffer A.

RESULTS

Distribution of 5′-Nucleotidases—5′-Nucleotidase, as well as alkaline phosphatase, activity was observed with both the culture supernatant and washed cells of *B. subtilis* K No. 231 cultured in a medium containing 0.2 or 1 g per liter of potassium phosphate (low or high phosphate medium, respectively), as shown in Table I. When the bacterium was grown in the low phosphate medium, 5′-nucleotidase activity in the supernatant was twice that in the cells. Increase of the phosphate concentration in the culture medium caused decreases of the activities of both fractions, but the effect was much more marked in the supernatant than in the cells. The results suggest that forma-

TABLE I. Distribution of 5′-nucleotidases and alkaline phosphatases in the culture supernatant and cells. *B. subtilis* K No. 231 was cultured in Medium-2 (high-phosphate medium) or Medium-3 (low-phosphate medium) for 48 h, then the cells were separated by centrifugation at 30,000×*g* for 20 min. The washed cells (381 mg dry weight from Medium-2 or 334 mg from Medium-3) were suspended in 10 ml of buffer A and ruptured with a sonic oscillator (Toyoriko type N-50-3, 10 kc) until complete disruption of the cells was confirmed by microscopic observation (for 10–15 min). The sonicate was centrifuged at 30,000×*g* for 30 min and the precipitates (cell debris) were washed twice with the same buffer then treated with 20 mg of lysozyme at 30°C for 30 min. The mixture was centrifuged at 30,000×*g* for 30 min. 5′-Nucleotidase activity of each fraction was assayed by method A in the presence or absence of 10 mM $MgCl_2$.

Fraction	Enzyme activities (units×10^3/mg cell)					
	Low-phosphate medium			High-phosphate medium		
	5′-Nucleotidase		Alkaline phosphatase	5′-Nucleotidase		Alkaline phosphatase
	None	10 mM $MgCl_2$		None	10 mM $MgCl_2$	
Culture supernatant	189	282	287	54	63	29
Cells	88	96	383	57	70	1.6
Sonicate	96	115	439	65	82	—
Supernatant	14	27	87	6.5	16	—
Precipitate (cell debris)	59	71	307	44	52	—
Lysozyme treatment of cell debris						
Soluble fraction	52	54	3.3	28	30	—
Precipitates	7.9	7.7	309	7.6	6.6	—

tion of the cellular and extracellular 5′-nucleotidase may be independently repressed by inorganic phosphate. Much stronger repression was observed with alkaline phosphatase than with 5′-nucleotidase.

When the cells were subjected to sonic treatment, slight but significant increases (10 to 20%) of both enzyme activities were observed. Since the substrates, phosphate esters, do not appear to be able to permeate through the cell surface, the results suggest that both enzymes may be located mostly on the surface of the cells, but partly inside the cells.

In a separate experiment, the washed cells were treated with lysozyme in the presence of 20% sucrose to prepare spheroplasts, and activities of 5′-nucleotidase, alkaline phosphatase and glucose-6-phosphate dehydrogenase released were assayed, as shown in Fig. 1. After treatment for 50 min, 82% of 5′-nucleotidase of the cells was released, whereas alkaline phosphatase, which has been reported to be located on the cell membrane (*10*), was not released during this incubation period. Moreover, glucose-6-phosphate dehydrogenase, which is thought to be a cytoplasmic enzyme, was not released significantly, indicating that spheroplasts formed did not burst during the incubation. Therefore, the results in Fig. 1 suggest that most of the 5′-nucleotidase of the cells may be located outside the cytoplasmic membrane, whereas alkaline phosphatase may be bound with the membrane, as reported by Le Hègarat *et al.* (*10*).

Upon centrifugation of the sonically disrupted cells at 30,000×*g*, the supernatant contained only 10 to 23% of the 5′-nucleotidase activity of the original sonicate (Table I). The time course of the sonic extraction, shown in Fig. 2, indicates that 5′-nucleotidase is gradually released up to about 10% of the total activity of the cells during the first 5 min of sonic treatment, but no subsequent release was observed. The time course of release of glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme, as well as microscopic observation of the sonicate, showed that complete rupture of the cells had been achieved during the first 5 min of the treatment. The results indicate that the 5′-nu-

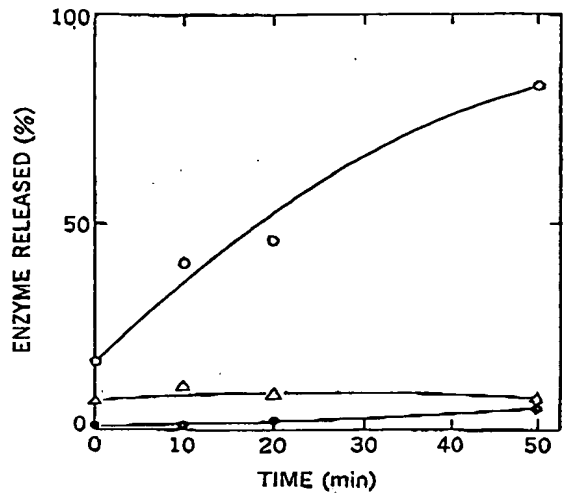

Fig. 1. Release of 5′-nucleotidase during spheroplast formation by lysozyme treatment. The washed cells (116 mg dry weight) of *B. subtilis* K No. 231, which had been grown in Medium-3 for 48 h, were incubated with 20 mg of lysozyme in buffer B in a total volume of 10 ml at 30°C. At the indicated times, 2-ml aliquots were pipetted into 2 ml of buffer B at 0°C, and the mixture was centrifuged at 15,000 × *g* for 10 min. The supernatant was removed and the residue was gently suspended in 3 ml of buffer B and centrifuged again. The pellet was suspended in 3 ml of buffer A and subjected to sonic disruption (10 kc) for 5 min. The sonicate (1.5 ml) was centrifuged at 30,000 × *g* for 30 min. 5′-Nucleotidase and alkaline phosphatase activities were assayed with the supernatant fluid from the incubation mixture and with the sonicate, while glucose-6-phosphate dehydrogenase was assayed with the supernatants obtained from the incubation mixture and from the sonicate. Total activity was obtained by summing the activities measured with the two fractions. 5′-Nucleotidase (○); alkaline phosphatase (△); glucose-6-phosphate dehydrogenase (●).

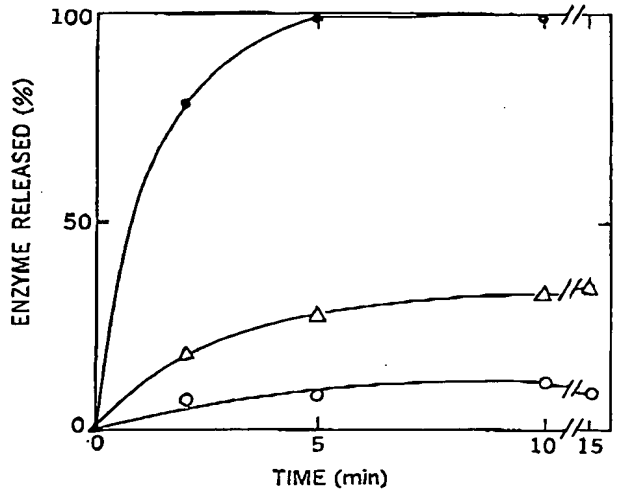

Fig. 2. Release of 5′-nucleotidase during sonic treatment of the cells. Washed cells (516 mg dry weight) of *B. subtilis* K No. 231 grown in Medium-2 were suspended in 30 ml of buffer A and ruptured with a sonic oscillator (10 kc). At the indicated times, 2-ml aliquots were removed and centrifuged at 30,000 × *g* for 30 min. The pellet was washed once with buffer A and suspended in 2 ml of the same buffer. 5′-Nucleotidase and alkaline phosphatase were assayed in the supernatant and the pellet obtained after sonic treatment. The total activities of the sonicate were obtained by summing the activities measured in both fractions. Glucose-6-phosphate dehydrogenase was assayed only in the supernatant. The maximum activity obtained during sonic treatment was used as the total activity of the sonicate. 5′-Nucleotidase (○); alkaline phosphatase (△); glucose-6-phosphate dehydrogenase (●).

cleotidase released may be a different enzyme species from that which associates with cell debris. This soluble enzyme appears to be located in the cytoplasmic space, because the amount of the activity was approximately equal to that of the increase during sonic treatment of the cells.

As shown in Table I, the sum of 5′-nucleotidase activities of the sonicate supernatant and precipitates was less than that of the original sonicate. In a separate experiment with the sonicate, using sonicate supernatant and precipitates having specific activities (units × 10^3/mg cell) of 96, 13, and 61, respectively, mixing of the last two fractions produced an activity of 94. This result suggests that there may be some activator(s) in the supernatant and, therefore, that the activity of precipitates may be lower than expected, because of the absence of the activators. Therefore, the actual 5′-nucleotidase activity of the precipitates (cell debris) in the sonicate may be regarded as the value obtained by subtracting the activity of the sonicate supernatant from that of the sonicate. Thus, the fractional value of the activity for the cell debris in the sonicate was calculated to be 86 and 90% of the total activity of the sonicate for cells grown in Medium-3 and Medium-2, respectively.

When the sonicate precipitates (cell debris) were treated with various kinds of inorganic salts, detergents and hydrolytic enzymes, as shown in

TABLE II. Solubilization of 5′-nucleotidase and alkaline phosphatase from the cell debris by various reagents. Cell debris, which was prepared by sonic treatment from cells grown in Medium-3, as described in Table I, was incubated with the indicated concentrations of the reagents at a concentration corresponding to 28 mg/ml of the dry cells in buffer A at 37°C for 30 min. Then, the mixture was centrifuged at 30,000×*g* for 15 min. Solubilization of 5′-nucleotidase and alkaline phosphatase was determined by measuring both enzyme activities of the mixture (total activity) and of the supernatant.

Reagent	Concentration	5′-Nucleotidase		Alkaline phosphatase	
		Total activity (unit)	Solubilized activity (%)	Total activity (unit)	Solubilized activity (%)
None		1.47	3.9	0.73	3.2
NaCl	2 M	1.37	15	0.47	51
$MgCl_2$	1 M	1.76	17	0.40	79
Sodium dodecyl sulfate	0.1%	1.27	7.0	1.21	33
Deoxycholate	1%	1.49	5.3	0.74	39
Triton X-100	1%	1.40	2.8	0.89	7.5
Tween 60	1%	0.91	3.9	0.78	0.6
Lysozyme	2 mg/ml	1.49	96	0.63	21
Chymotrypsin	0.5 mg/ml	1.45	2.7	2.38	11
Trypsin	0.5 mg/ml	1.31	16	1.93	11
Subtilisin	0.5 mg/ml	1.20	11	1.69	3.5
Phospholipase C	0.5 mg/ml	1.48	14	0.89	12
Ribonuclease	0.05 mg/ml	1.41	3.4	0.69	2.6
Deoxyribonuclease	0.05 mg/ml	1.39	4.7	0.68	2.7

Table II, only lysozyme specifically solubilized 5′-nucleotidase (96%) from the cell debris, while alkaline phosphatase was solubilized by 1 M $MgCl_2$, 2 M NaCl or some detergents. Since lysozyme is known to hydrolyze the peptidoglycan of bacterial cell wall specifically (*11*), the present results suggest that 5′-nucleotidase of the cell debris fraction may bind with the cell wall, in contrast to alkaline phosphatase (*10*). This suggestion is also supported by the observation that almost all the cellular 5′-nucleotidase is released during spheroplast formation, as shown in Fig. 1. From the solubilization data of Tables I and II, approximately 90% of 5′-nucleotidase in the cell debris fraction or 80% of the cellular enzyme seems to bind with the cell wall of cells grown in the low phosphate medium.

In order to determine whether 5′-nucleotidase is located in the cytoplasm and cytoplasmic membrane, spheroplasts were prepared from cells grown in Medium-3 and burst by osmotic shock. The 5′-nucleotidase activity of the burst spheroplasts

TABLE III. Distribution of 5′-nucleotidases in spheroplasts. Experimental conditions are given in "MATERIALS AND METHODS." Under these conditions, 98% of cells grown in Medium-3 were converted into spheroplasts. The enzyme activities of each fraction were corrected for the incomplete conversion. The 5′-nucleotidase activity of each fraction was assayed by method A in the presence or absence of 10 mM $MgCl_2$.

Fraction	5′-Nucleotidase activity (units×10^2/mg cell)	
	None	10 mM $MgCl_2$
Cells	84	92
Lysozyme treatment	101	119
Supernatant at 15,000×*g*	67	78
Spheroplast burst	18	25
Supernatant at 30,000×*g* of the burst spheroplasts	7.8	15
Precipitate at 30,000×*g* of the burst spheroplasts	6.9	7.6

was 18% of the total activity of the original lysozyme-treated cells, as shown in Table III. Upon centrifugation of the burst spheroplast fraction, 43% and 38% of the enzyme activity was observed in the supernatant, which can be regarded as the cytoplasmic fraction, and in the washed precipitates, which can be regarded as the cytoplasmic membrane fraction, respectively. This indicates that at least 8% and 7% of the cellular 5′-nucleotidases exist in the cytoplasm and on the cytoplasmic membrane, respectively. In the experiments shown in Table I, the precipitates obtained by centrifugation after lysozyme treatment of the sonic cell debris from the cells grown in Medium-3 and Medium-2 were thought to be cytoplasmic membrane fragments, which showed 11% and 8% of the 5′-nucleotidase activity of the original sonicates, respectively. The latter value is in good agreement with that for the precipitate fraction of the burst spheroplasts, as described above (Table III). Upon further centrifugation of the supernatant of burst spheroplasts at 100,000 × *g* for 60 min, most of the enzyme activity was observed in the supernatant, indicating that most of the cytoplasmic 5′-nucleotidase exists in a soluble state.

The cytoplasmic 5′-nucleotidase was activated about two-fold in the presence of 10 mM $MgCl_2$, whereas the cell wall and membrane enzymes were activated only slightly, as shown in Tables I and III, respectively. Supernatants of the cell sonicates were also activated two-fold by 10 mM $MgCl_2$, as shown in Table I. These results support the above suggestion that 5′-nucleotidase in the sonicate supernatant may be cytoplasmic. As shown in Table I, among the three cellular 5′-nucleotidase enzymes, the cytoplasmic (sonicate supernatant) enzyme was the most repressible by inorganic phosphate.

Purification of 5′-Nucleotidase—*B. subtilis* K No. 231 was grown in Medium-2 or -3 for 48 h, then cellular and extracellular 5′-nucleotidases were purified from the culture supernatant and the washed cells, respectively. The results are summarized in Table IV. Two enzymatically active peaks were observed on a DEAE-cellulose column chromatogram of the dialyzed culture supernatant, as shown in Fig. 3A. The enzyme of the first peak (E_1) passed through the column during washing, while that of the second peak (E_2) was eluted at about 0.05 M NaCl. The two enzymes were further purified by gel filtration separately on Sephadex G-100. The final E_1 and E_2 preparations showed 54- and 70-fold higher specific activities, respectively, than the dialyzed culture supernatant.

To purify the cytoplasmic enzyme, the cells were ruptured by sonic treatment and the sonicate was fractionated by centrifugation into the sonicate supernatant and the cell debris. The supernatant fraction was further fractionated with ammonium sulfate, then gel-filtered through Sephadex G-200, and finally subjected to DEAE-cellulose column chromatography, yielding two separate active peaks of 5′-nucleotidase, as shown in Fig. 3B. The pooled enzyme preparations of the first and second peaks (C_1 and C_2, respectively) showed 17- and 33-fold higher specific activities than the sonicate supernatant. Similar preparations were also obtained from supernatant of burst spheroplast.

5′-Nucleotidase of the cell wall was solubilized by lysozyme treatment of the cell debris, as described above. As shown in Fig. 3C, three peaks of the enzyme activity were observed on a DEAE-cellulose column chromatogram of the solubilized cell-wall fraction which was previously fractionated with ammonium sulfate and then gel-filtered through Sephadex G-200. The pooled enzyme preparations from the first, second and third peaks (W_1, W_2, and W_3, respectively) showed 65-, 42- and 134-fold higher specific activities, respectively, than the solubilized cell-wall fraction.

Molecular Weight—The molecular weights of 5′-nucleotidase in the seven different preparations E_1, E_2, W_1, W_2, W_3, C_1, and C_2, were determined by gel filtration with Sephadex G-100 or G-200 to be 27,000, 46,000, 37,000, 21,000, 640,000, 38,000, and 260,000, respectively. In contrast, the elution profile of the enzyme activity of the dialyzed culture supernatant before DEAE-cellulose column chromatography showed a single enzymatically active peak with a molecular weight of 67,000. This value is larger than those of E_1 and E_2. Similarly, before DEAE-cellulose column chromatography, the enzymes from the cell wall and the sonicate supernatant also gave single peaks with higher molecular weights, about 900,000 and 600,000 respectively, than those of the respective purified preparations, W_1, W_2, W_3, C_1, and C_2. The results indicate that the culture supernatant,

TABLE IV. Purification of 5′-nucleotidase from culture supernatant and cells of *B. subtilis*. Experimental conditions are given in " MATERIALS AND METHODS."

Purification step	Total volume (ml)	Total protein (mg)	5′-Nucleotidase	
			Total activity (units)	Specific activity (units/mg protein)
Culture supernatant	4,000	4,200	460	0.11
DEAE-cellulose				
peak E_1	7.5	14	53	3.8
peak E_2	5	12	32	2.6
Sephadex G-100				
E_1	38	1.9	11.7	6.0
E_2	25	0.6	5.1	7.7
Sonicate supernatant	237	3,300	103	0.03
First $(NH_4)_2SO_4$	23.5	1,300	61	0.10
Second $(NH_4)_2SO_4$	3.6	260	28	0.35
Sephadex G-200	35	38	14	0.35
DEAE-cellulose				
C_1	44	3.2	1.6	0.50
C_2	32	12	1.2	1.00
Sonicate precipitate solubilized by lysozyme	207	1,100	300	0.26
$(NH_4)_2SO_4$	8.2	44	280	0.64
Sephadex G-200	34.5	13	150	11.7
DEAE-cellulose				
W_1	36	1.2	25	17
W_2	40	1.1	12	11
W_3	28	0.4	14	35

cell wall, and cytoplasm originally each contain one kind of 5′-nucleotidase, which may be dissociated into smaller molecular species during subsequent DEAE-cellulose chromatography. In addition, addition of 0.2 M $MgCl_2$ to the buffer system for gel filtration also caused a decrease in the molecular weight of the crude preparations of cell wall and cytoplasmic enzymes.

Optimum pH—All the enzymes had optimum pH's between 7.0 and 8.0, when the reactions were carried out by assay method A at pH's from 4.0 to 10.5 using 0.1 M sodium acetate, TES-NaOH, Tris-HCl, or glycine-NaOH buffer.

Substrate Specificity—All the enzymes specifically hydrolyzed purine nucleoside-5′-monophosphates but not 5′-pyrimidine nucleotides, as shown in Table V. Among the nucleotides tested, 5′-IMP was the best substrate. Cellular enzymes (C_1, C_2, W_1, W_2, W_3) showed slight activities toward UDP-glucose, whereas the extracellular enzymes (E_1, E_2) did not. None of the enzymes, except for C_1, hydrolyzed 4-nitrophenylphosphate or bis(4-nitrophenyl)phosphate at pH 10 or at pH 7.2 (data at pH 7.2 are not shown). Judging from the low specific activity of the C_1 preparation, as shown in Table IV, the activities of C_1 toward the two synthetic compounds may be due to contaminating enzymes. On the other hand, alkaline phosphatase in *B. subtilis* K seems to have no significant 5′-nucleotidase activity at pH 7.2, based on the following observation: when the bacterium was grown in a high-phosphate medium,

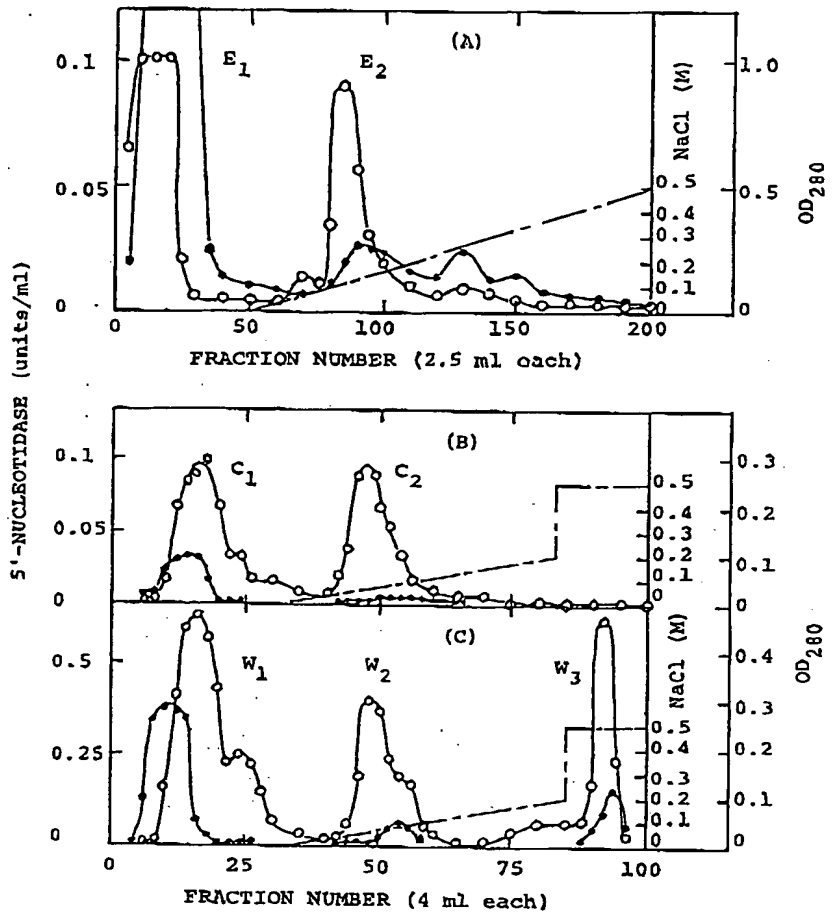

Fig. 3. DEAE-cellulose chromatography of 5'-nucleotidases of the culture supernatant (A), cell sonicate supernatant (B) and solubilized cell-wall fraction (C). (A): *B. subtilis* K No. 231 was grown in Medium-3 for 48 h. Culture supernatant (50 ml) was dialyzed against 1 liter of buffer A overnight. After removal of insoluble materials by centrifugation, the supernatant was introduced into a DEAE-cellulose column (1.5×20 cm) previously equilibrated with buffer A. After the sample had passed through the column, the column was washed with 70 ml of the same buffer, and eluted with a concentration gradient of NaCl formed from 200 ml of buffer A and 200 ml of the same buffer containing 0.5 M NaCl. (B) and (C): Pooled fractions after Sephadex G-200 chromatography of the sonicate supernatant (35 ml) or of solubilized cell-wall fraction (35 ml) were introduced into DEAE-cellulose columns (1.5×15 cm) previously equilibrated with buffer A. After the sample had passed through the column, the column was washed with 100 ml of the buffer, and eluted with a concentration gradient of NaCl formed from 100 ml of buffer A and 100 ml of the buffer containing 0.2 M NaCl. Elution was continued by using 50 ml of the buffer containing 0.5 M NaCl. 5'-Nucleotidase (○); OD_{280} (●); NaCl (—·—).

alkaline phosphatase activity was mostly present in the culture supernatant (Table I), from which more than 95% of the activity was precipitated during dialysis against 0.05 M Tris-HCl buffer, pH 7.5. After being washed twice with 0.4 M NaCl, this precipitate showed a 5'-nucleotidase activity of only 0.0073 (pH 7.2) relative to the activity toward 4-nitrophenylphosphate (pH 10).

TABLE V. Substrate specificity of 5′-nucleotidases. The activities for all the nucleotides were followed by assay method A, except that 10 mM substrate was used with E_1 (4.8 μg as protein), E_2 (15 μg), C_1 (36 μg), C_2 (18 μg), W_1 (6.6 μg), W_2 (5.4 μg), or W_3 (2.8 μg) as the enzyme. The activities for 4-nitrophenylphosphate and bis(4-nitrophenyl)phosphate were followed by the method used for alkaline phosphatase, except that 10 mM substrate was used and the reaction was carried out for 30 min.

Substrate (10 mM)	Relative activity (%)						
	5′-Nucleotidase						
	E_1	E_2	C_1	C_2	W_1	W_2	W_3
5′-IMP	100	100	100	100	100	100	100
5′-AMP	53	62	58	49	60	59	59
5′-GMP	68	77	69	70	49	79	79
5′-XMP	99	102	81	88	72	83	79
5′-CMP	9	8	0	0	1	2	10
5′-UMP	4	8	8	3	6	14	12
5′-ATP	0	0	2	27	0	4	4
Glucose-1-P	0	1	4	10	5	4	0
UDP-glucose	0	0	15	32	8	33	17
ADP-glucose	—	—	6	6	0	7	3
4-Nitrophenylphosphate	0	0	19	0	0	0	0
Bis(4-nitrophenylphosphate)	1	2	62	11	5	7	3

This means that, even if alkaline phosphatase has some 5′-nucleotidase activity, it amounts to less than 0.4% of the total 5′-nucleotidase activity of the culture supernatant.

Effects of Various Compounds—High concentrations (0.1 and 1.0 M) of inorganic salts, including NaCl, KCl, and $(NH_4)_2SO_4$, activated the cellular 5′-nucleotidase more strongly than the extracellular enzymes, while Zn^{2+} and Cu^{2+} inhibited the former more strongly than the latter, as shown in Table VI. On the other hand, Mg^{2+} and Mn^{2+} stimulated the activities of the cytoplasmic enzymes, C_1 and C_2, specifically. All the enzymes were strongly inhibited by ATP. The inhibitory effect of inorganic phosphate was less strong and was only partial, with a maximum value of about 60%, at more than 1 M. This means that the binding site of phosphate as an inhibitor must be different from the catalytic site on the enzymes, and that the enzyme-AMP-phosphate complex still has some activity.

Kinetic Properties—Double-reciprocal plots of the reaction rate against the substrate (AMP) concentration showed typical Michaelis-Menten kinetics with all seven 5′-nucleotidases, giving almost the same K_m values for AMP; 1.5, 1.5, 1.5, 1.9, 1.7, 1.7, and 1.5 μM for E_1, E_2, C_1, C_2, W_1, W_2, and W_3, respectively. Figure 4 shows the results with E_1 and E_2 as examples. The figure also shows that the inhibition by phosphate is of mixed type with respect to AMP. From the results of the mixed-type inhibition and of the partial inhibition (Table VI), and assuming rapid equilibrium, the following rate equation was derived.

$$v=\frac{V+V'(K_m/K_m')(I/K_1)}{1+(K_m/K_m')(I/K_1)+(K_m/S)(1+1/K_1)} \quad (1)$$

where S, I, K_m, K_m', K_1, V, and V' are the concentrations of AMP and PO_4^{2-}, Michaelis constants for AMP of enzyme-AMP complex and enzyme-PO_4^{2-}-AMP complex, inhibitor constant for PO_4^{2-} of enzyme-PO_4^{2-} complex, and the maximum velocities of the reactions in the absence and presence of PO_4^{2-}, respectively. The base-line intercepts, r, of the double-reciprocal plots in Fig. 4 are given as a function of I as follows,

TABLE VI. Effects of inorganic salts and ATP on 5′-nucleotidases. The reaction, except in the case of KH_2PO_4, was performed by assay method A, except that the indicated concentrations of the compounds described in the table and 5′-nucleotidase E_1, E_2, C_1, C_2, W_1, W_2, or W_3 (the same amount of protein as in Table V) were added to the reaction mixtures. The reaction with KH_2PO_4 was performed by assay method B, except that the indicated concentrations of KH_2PO_4 and 5′-nucleotidase E_1 (1.2 μg as protein), E_2 (3.8 μg), C_1 (9 μg), C_2 (5 μg), W_1 (1.4 μg), W_2 (1.3 μg), or W_3 (0.7 μg) were added to the reaction mixtures.

Compound and concentration		Relative activity (%)						
		5′-Nucleotidase						
	(mM)	E_1	E_2	C_1	C_2	W_1	W_2	W_3
None		100	100	100	100	100	100	100
NaCl	1,000	110	90	170	200	250	—	120
	100	100	90	—	130	150	—	100
	10	100	110	—	100	110	—	120
KCl	1,000	120	110	190	230	330	—	150
	100	100	110	—	110	180	—	110
	10	100	100	—	120	130	—	80
$(NH_4)_2SO_4$	1,000	230	190	370	490	550	—	320
	100	130	130	—	230	260	—	150
	10	120	120	—	120	120	—	110
$MgCl_2$	10	100	90	160	180	110	110	100
	1	100	100	130	120	110	110	80
$MnCl_2$	4	90	90	150	140	120	100	100
	1	100	100	130	120	120	110	90
$CaCl_2$	10	90	100	130	120	120	120	110
	1	100	100	130	120	90	100	110
$ZnCl_2$	1	30	20	0	0	10	6	8
$CuCl_2$	1	60	60	0	0	20	4	20
$CoCl_2$	10	30	30	70	40	30	—	30
	1	90	100	90	70	50	60	80
$FeSO_4$	1	120	140	70	190	50	250	220
KH_2PO_4	400	40	40	—	—	—	—	—
	100	40	40	—	—	—	—	—
	10	50	50	60	50	40	40	40
	1	80	80	70	70	70	70	80
ATP	10	0	0	0	0	10	7	8
	1	30	30	8	0	20	30	20
	0.1	60	—	40	30	50	50	60

$$\frac{1}{r_0 - r} = \frac{K_m K_m'}{K_m' - K_m}(1 + K_I/I) \qquad (2)$$

where r_0 is r at I=0. Thus, K_I values for E_1 and E_2 were calculated to be 1.0 mM and 1.1 mM, respectively, from the secondary plots shown in the *inserts* of Fig. 4 based on Eq. 2. The results (data not shown) with the five cellular enzymes

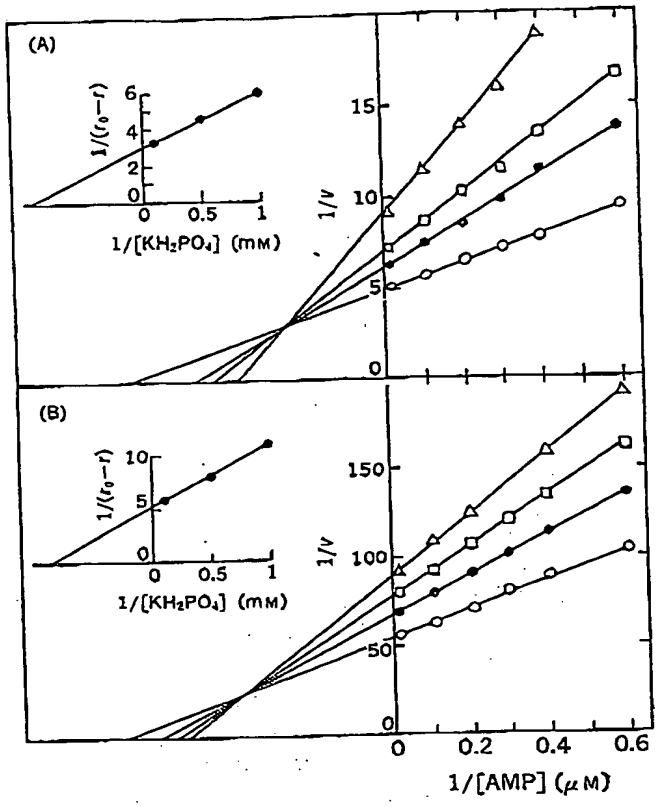

Fig. 4. Double-reciprocal plots of reaction velocity against AMP concentration in the presence and absence (○) of KH_2PO_4. The reaction was performed by assay method B except that the indicated concentrations of AMP, 1 mM KH_2PO_4 (●), 2 mM KH_2PO_4 (□), or 10 mM KH_2PO_4 (△), and 5′-nucleotidase E_1 (1.2 μg as protein) (A) or E_2 (3.8 μg) (B) were added to the reaction mixtures. v, r, and r_0: see the text.

also showed mixed-type inhibition by PO_4^{2-}, with almost the same K_I values; 0.4, 0.7, 0.8, 1.1, and 0.9 mM, for C_1, C_2, W_1, W_2, and W_3, respectively.

DISCUSSION

From the present results, it is clear that 5′-nucleotidases of *B. subtilis* exist in the extracellular medium, cell wall, cytoplasm and cytoplasmic membrane. A cell-surface localization of 5′-nucleotidase has been reported for *E. coli* (*12*), *B. subtilis* MB-1839 (*5*), W-23 (*7*), Marburg (*13*), *B. cereus* (*14*), and *Xanthomonas oryzae* (*15*). Furthermore, Heppel (*16*) and Neu (*12*) reported that 5′-nucleotidase of *E. coli* was located in the periplasmic space. Mauck and Glaser (*7*) also reported that nucleoside diphosphate sugar hydrolase having 5′-nucleotidase activity was located in the periplasmic space in *B. subtilis* W-23. However, 5′-nucleotidases bound to the cell wall as well as the cytoplasmic enzyme described in the present paper have now been observed for the first time in microorganisms. The finding of cytoplasmic 5′-nucleotidase was also supported by the following result (data will be published elsewhere) with a mutant having very low 5′-nucleotidase activity: washed intact cells of the mutant grown in Medium-2 did not show any 5′-nucleotidase activities in the presence of $MgCl_2$, whereas significant activity was observed after sonic disruption of the cells. It is not clear from the present study whether or not the enzyme exists in the periplasmic space of *B. subtilis* K, because no specific method for isolating periplasmic enzyme is available when cell-wall bound enzyme is present.

None of the 5′-nucleotidase preparations, except for C_1, hydrolyzed 4-nitrophenylphosphate or bis(4-nitrophenyl)phosphate at pH 10. On the other hand, extracellular alkaline phosphatase was found not to hydrolyze 5′-nucleotides at pH 7.2. Therefore, it can be concluded that non-specific alkaline phosphatase and 5′-nucleotidase may be different entities.

As described above, nucleoside diphosphate sugar hydrolase isolated and purified from *B. subtilis* W-23 cells has been found to have 5′-nucleotidase activity (*7*). The present results also show that some of the cellular 5′-nucleotidases hydrolyzed UDP-glucose.

5′-Nucleotidases from *B. subtilis* K specifically hydrolyze purine nucleoside-5′-monophosphate, but not pyrimidine nucleotides, in contrast to other bacterial 5′-nucleotidases so far reported (*7*, *12*, *14*, *15*, *17*), which all hydrolyze both purine and pyrimidine nucleotides. Although all seven enzyme preparations from this bacterium showed similar enzymatic properties, including optimum pH, substrate specificity, Michaelis constant (K_m) for AMP and inhibitor constant (K_I) for PO_4^{2-}, their responses to inorganic salts were somewhat different; the extracellular enzymes (E_1 and E_2) showed almost no activation by high concentrations of NaCl or KCl, while the cytoplasmic enzyme, C_1 and C_2, were specifically activated by Mg^{2+} or Mn^{2+}.

As regards the regulation of 5′-nucleotidase,

both the cellular and extracellular enzyme activities per unit cell weight clearly depended on the phosphate concentration in the culture medium. Inorganic phosphate was also an allosteric inhibitor of the enzymes.

Regarding the metabolic role of the cytoplasmic enzymes, C_1 and C_3, these enzymes appear to be involved in the extracellular accumulation of purine nucleosides in *B. subtilis* K, since they are located in the cytoplasm, and the cell membrane acts as a permeability barrier to the excretion of 5′-nucleotides formed inside cells. Utilization of 5′-nucleotides in the extracellular medium may involve the other five enzymes, since they are all located on the outside of the cell membrane.

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